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Claims for the following Contracting State: AT + ES.

(54) Site-specific recombination of DNA in yeast.

(57) A method for producing site-specific recombination of DNA in yeast at regions designated lox sites is disclosed. Novel strains of yeast transformed with DNA sequences comprising two lox sites, a regulatory nucleotide sequence and a cre gene and yeast-transforming plasmids having a regulatory nucleotide sequence and a cre gene are also disclosed.

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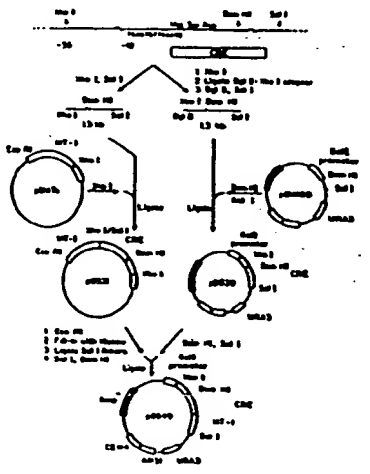

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Description

Site-Specific Recombination of DNA in Yeast

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to a method for producing site-specific recombination of DNA in yeast.

Background of the Invention

Yeast are a promising host for commercial applications of genetic engineering. A method for producing site-specific recombination of DNA in yeast would enhance the commercial potential of yeast as hosts for genetically engineered products.

Abremski et al., Cell, 32: 1301-1311 (1983) disclose a site-specific recombination system of bacteriophage P1. The system consists of a recombination site designated loxP and a recombinase designated Cre. The authors show that recombination between loxP sites on supercoiled, nicked-circle or linear DNA occurs in the presence of Cre.

Brent et al., Nature, 312: 612-615 (1984) disclose that a bacterial repressor protein or a yeast transcriptional terminator can block upstream activation of a yeast gene. Disclosed experiments are said to provide genetic evidence that a bacterial repressor protein manufactured in the yeast cytoplasm can enter the yeast nucleus, recognize its operator and repress gene transcription from a yeast promoter.

Barnes et al., Proc. Natl. Acad. Sci 82: 1354-1358 (1985) disclose that the bacterial restriction enzyme Eco RI able to enter and function within the nucleus of Saccharomyces cerivisiae when the procaryotic protein is synthesized in vivo.

Backman et al., Bio/Technology (December, 1984) disclose a site-specific recombination system of the bacteriophage lambda. The system catalyzes recombination between two different sites in DNA, designated attP and attB, to yield two other different sites, designated attR and attL, or vice versa. Recombination occurs only in the presence of certain E. coli proteins and the Int protein of bacteriophage lambda and can be used to regulate gene expression of E. coli. Langeveld et al., Mol. Gen. Genet., 199:396-400 (1985) disclose expression of an E. coli phr gene in yeast Saccharomyces cerevisiae.

Summary of the Invention

The present invention provides a method for producing site-specific recombination of DNA in yeast. The method comprises introducing into the DNA the following sequences:

a) a first DNA sequence comprising a regulatory nucleotide sequence and a cre gene,
b) a second DNA sequence comprising a first lox site, and
c) a third DNA sequence comprising a second lox site.

The regulatory nucleotide sequence is activated thereby effecting expression of the cre gene and producing the site-specific recombination. In a preferred embodiment, the second and third DNA sequences are inserted into DNA in yeast connected by a pre-selected DNA segment. There follows herein a brief description of the drawings and a general description of the invention.

Figure 1 represents the construction of plasmids pBS39 and pB149, containing the GAL1 promoter and the cre gene.

Figure 2 represents the construction of plasmids pBS42 and pBS43, containing a functional LEU2 gene flanked by lox sites in the same orientation. The lox sites are indicated by ▮▶.

Figure 3 represents the modification of chromosome 7 of yeast strain DBY931 after homologous recombination with pBS42 (panel A) or pBS43 (panel B). The lox sites are indicated by ▮▶. The centromere is indicated by ●.

Figure 4 shows the deletion of the LEU2 gene from yeast strain BSY38 after activation of the GAL1 promoter as described in Example 1.

Figure 5 shows that the deletion of the LEU2 gene occurs at the lox sites, as described in Example 1. The lox sites are indicated by ▮▶; Eco RI sites are indicated by ↑. The distance between Eco RI sites is indicated in kilobases (kb).

Figure 6 represents the modification of chromosome 13 of yeast strain DBY931 after homologous recombination with pBS44 (panel A) or pBS47 (panel B), as described in Example 3.

The present invention provides a method for producing site-specific recombination of DNA in yeast. DNA sequences comprising a cre gene and first and second lox sites are introduced into the DNA and expression of the cre gene produces recombination at the lox sites. The location and orientation of the lox sites determines the nature of the recombination.

As used herein, the expression "site-specific recombination" is intended to include the following three events:

1. deletion of a pre-selected DNA segment flanked by lox sites,

2. inversion of the nucleotide sequence of a pre-selected DNA segment flanked by lox sites, and

3. reciprocal exchange of DNA segments proximate to lox sites located on different DNA molecules.

"DNA segment" refers to a linear fragment of single- or double-stranded deoxyribonucleic acid (DNA), which can be derived from any source. The expression "DNA in yeast" includes all DNA present in yeast cells. As used herein, a "gene" is intended to mean a DNA segment which is normally regarded as a gene by those skilled in the art. The expression "regulatory molecule" refers to a polymer of ribonucleic acid (RNA) or a polypeptide which is capable of enhancing or inhibiting expression of a gene.

"Regulatory nucleotide sequence", as used herein, refers to a nucleotide sequence located 5' to a gene whose transcription is controlled by the regulatory nucleotide sequence in conjunction with the gene expression apparatus of the cell. The expression "nucleotide sequence" refers to a polymer of DNA or RNA, which can be single- or double-stranded, optionally containing synthetic, non-natural, or altered nucleotides capable of incorporation into DNA or RNA polymers. As used herein, a "regulatory nucleotide sequence" can include a promoter region, as that term is conventionally employed by those skilled in the art. A promoter region can include an association region recognized by an RNA polymerase, one or more regions which control the effectiveness of transcription initiation in response to physiological conditions, and a transcription initiation sequence. "Gene product" refers to a polypeptide resulting from transcription, translation, and, optionally, post-translational processing of a selected DNA segment.

In the present method, a first DNA sequence comprising a regulatory nucleotide sequence and a cre gene is introduced into DNA in yeast. Suitable regulatory nucleotide sequences include GAL1, GAL10, ADH1, CYC1, and TRP5 promoters. GAL1 and GAL10 promoters are present on plasmid pBM150 which is described by Johnston and Davis, Molec. Cell. Biol., 4:1440 (1984). The ADH1 promoter, also called ADC1, is present on plasmid pAAH5 which is described by Ammer, Methods Enzymol., 101:192 (1983). The CYC1 promoter is described by Stiles et al., Cell, 25:277 (1981). The TRP5 promoter is described by Zalkin and Yanofsky, J. Biol. Chem., 257:1491 (1982). Preferably, the regulatory nucleotide sequence is a GAL1 promoter.

The gene product of the cre gene is a recombinase herein designated "Cre" which effects site-specific recombination of DNA in yeast at lox sites. As used herein, the expression "cre gene" means a nucleotide sequence which codes for a gene product which effects site-specific recombination of DNA in yeast at lox sites. One cre gene can be isolated from bacteriophage P1 by methods known in the art. One method for isolating a cre gene from bacteriophage P1 is disclosed by Abremski et al., Cell, 32:1301-1311 (1983). E. coli DH1 and yeast strain pBSY90 transformed with plasmid pBS39 carrying a cre gene isolated from bacteriophage P1 and a GAL1 regulatory nucleotide sequence have been deposited with the American Type Culture Collection (ATCC) and bear deposit accession numbers ATCC 53255 and ATCC 20772, respectively. The cre gene can be isolated from plasmid pBS39 with restriction enzymes Xho I and Sal I.

Second and third DNA sequences comprising a first lox site and a second lox site, respectively, are also introduced into the DNA. As used herein the expression "lox site means a nucleotide sequence at which the gene product of the cre gene can catalyze a site-specific recombination. LoxP site is a 34 base pair nucleotide sequence which can be isolated from bacteriophage P1 by methods known in the art. One method for isolating a LoxP site from bacteriophage P1 is disclosed by Hoess et al., Proc. Natl. Acad. Sci. USA, 79:3398 (1982). The LoxP site consists of two 13 base pair inverted repeats separated by an 8 base pair spacer region. The nucleotide sequences of the insert repeats and the spacer region are as follows.

ATAACTTCGTATA ATGTATGC TATACGAAGTTAT

E. coli DH5 Δlac and yeast strain BSY23 transformed with plasmid pBS44 carrying two loxP sites connected with a LEU2 gene have been deposited with the ATCC and bear deposit accession numbers ATCC 53254 and ATCC 20773, respectively. The lox sites can be isolated from plasmid pBS44 with restriction enzymes Eco RI and Sal I, or Xho I and Bam I. In addition, a pre-selected DNA segment can be inserted into pBS44 at either the Sal I or Bam I restriction enzyme sites by techniques known in the art. Other suitable lox sites include LoxB, LoxL and LoxR sites which are nucleotide sequences isolated from E. coli. These sequences are disclosed and described by Hoess et al., Proc. Natl. Acad. Sci USA, 79:3398 (1982).

Preferably, the lox site is a LoxP site. Lox sites can also be produced by a variety of synthetic techniques which are known in the art Synthetic techniques for producing lox sites are disclosed by Ito et al., Nuc. Acid Res., 10:1755 (1982) and Ogilvie et al., Science, 214:270 (1981).

Methods for introducing DNA sequences into DNA in yeast at pre-selected regions are known in the art. Preferably, the DNA sequences are introduced by a plasmid capable of transforming yeast while carrying a DNA sequence. In one embodiment, the first, second, and third DNA sequences are introduced into one strain of yeast. Alternatively, the DNA sequences are introduced into two different strains of yeast of opposite mating types which are subsequently mated to form a single strain having all three DNA sequences. Preferably, the plasmid contains either (1) a nucleotide sequence of DNA homologous to a resident yeast sequence to permit integration into the yeast DNA by the yeast's recombination system or (2) a nucleotide sequence of DNA which permits autonomous replication in yeast. One nucleotide sequence which permits autonomous replication in yeast is an ARS sequence described by Stinchcomb et al., Nature, 282:39 (1979). A partial list of plasmids capable of transforming yeast includes YIP5, YRP17 and YEP24. These plasmids are disclosed and described by Botstein and Davis, The Molecular Biology of the Yeast Saccharomyces, Metabolism and Gene Expression (ed. Strathern et al.), (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), at page 607.

Most preferably, the plasmid for introducing a DNA sequence comprising a regulatory nucleotide sequence and a cre gene is pBS39 or pBS49 and the plasmid for introducing a DNA sequence comprising a lox site is pBS44, pBS47, pBS42, pBS43 or derivatives thereof carrying a pre-selected DNA segment other than or in addition to the LEU2 gene located between the first and second lox sites. E. coli DH1 and DH5 Δlac transformed with plasmids pBS39 and pBS44, respectively, have been deposited with the ATCC and bear deposit accession numbers 53255 and 53254, respectively. Yeast strains BSY90 and BSY23 transformed with plasmids pBS39 and pBS44, respectively, have also been deposited with the ATCC and bear deposit accession numbers ATCC 20772 and ATCC 20773, respectively. These yeast strains are opposite mating types and can be mated to form a single strain having plasmid pBS39 and a pBS44 modified chromosome. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

The lox site is an asymmetrical nucleotide sequence. Thus, two lox sites on the same DNA molecule can have the same or opposite orientations with respect to each other. Recombinations between lox sites in the same orientation result in a deletion of the DNA segment located between the two lox sites. The deleted DNA segment forms a circular molecule of DNA. The original DNA and the resulting circular molecule each contain a single lox site. Recombination between lox sites in opposite orientations on the same DNA molecule result in an inversion of the nucleotide sequence of the DNA segment located between the two lox sites. In addition, reciprocal exchange of DNA segments proximate to lox sites located on two different DNA molecules can occur. All of these recombination events are catalyzed by the gene product of the cre gene.

In a preferred embodiment of the present invention, the second and third DNA sequences are introduced into DNA in yeast connected by a pre-selected DNA segment. The segment can be a gene or any other sequence of deoxyribonucleotides of homologous, heterologous or synthetic origin. Preferably, the preselected DNA segment is a gene for a structural protein, an enzyme, or a regulatory molecule. If the first and second lox sites have the same orientation, activation of the regulatory nucleotide sequence produces a deletion of the pre-selected DNA segment. If the first and second lox sites have opposite orientation, activation of the regulatory nucleotide sequence produces an inversion of the nucleotide sequence of the pre-selected DNA segment.

UTILITY

Genes engineered into yeast for producing a foreign protein are often placed under the control of a highly active promoter. The activity of the promoter can result in an overproduction of the protein which interferes with the growth of the engineered yeast. This overproduction of the protein can make it difficult to grow engineered yeast in sufficient quantity to make protein production economically feasible. The present invention provides a method whereby engineered yeast can be grown to a desired density prior to expressing the engineered gene. The engineered gene is expressed, as desired, by activating a regulatory nucleotide sequence responsible for controlling expression of the cre gene. Methods of controlling the expression of an engineered gene according to the present invention include the following:

(1) A DNA segment flanked by lox sites in the same orientation is introduced into DNA in yeast between a promoter and an engineered gene to render the promoter incapable of expressing the gene. A second DNA sequence comprising a regulatory nucleotide sequence and a cre gene is also introduced in the DNA. After the engineered yeast are grown to a desired density, the regulatory nucleotide sequence is activated thereby effecting expression of the cre gene and producing a deletion of the DNA segment. The engineered gene would then be expressed.

(2) A gene for a regulatory molecule flanked by lox sites in the same orientation is introduced into DNA in yeast. The regulatory molecule inhibits expression of an engineered gene A second DNA sequence comprising a regulatory nucleotide sequence and a cre gene is also introduced into the DNA. After the engineered yeast are grown to a desired density, the regulatory nucleotide sequence is activated thereby effecting expression of the cre gene and producing a deletion of the gene for the regulatory molecule. The engineered gene would then be expressed.

(3) An engineered gene lacking a promoter and flanked by two lox sites in opposite orientations is introduced into DNA in yeast such that the 3' end of the gene lies adjacent to the transcription start site of a regulatory nucleotide sequence. A second DNA sequence comprising a regulatory nucleotide sequence and a cre gene is also introduced into the DNA. Since the engineered gene would be transcribed in the antisense direction, no engineered protein would be produced. After the engineered yeast is grown to a desired density, the regulatory nucleotide sequence is activated thereby effecting expression of the cre gene and producing an inversion of the desired gene. The engineered gene could then be transcribed in the proper direction and expressed.

Materials and Methods

Unless otherwise specified, parts and percentages used in this section are by weight and degrees are Celsius.

Strains and Media

E. coli strains DH1 and DH5 ΔlacU169, or a derivative thereof, served as the E. coli hosts for all plasmids used in all experiments. The DH5 ΔlacU169 strain was obtained from Dr. Michael Berman, Litton Bionetics, and is a derivative of DH5, a variant of DH1 which is disclosed and described by Hanahan, J. Mol. Biol., 166:557 (1983). Media used for bacterial growth are described in Maniatis et al., Molecular Cloning:A Laboratory Manual (Cold Spring Harbor Laboratory, New York, 1982).

The leu2 ura3 yeast strains DBY745 (mating type alpha) and DBY931 (mating type a) were used in all experiments. These strains are disclosed and described in Falco, Rose, and Botstein, Genetics, 105:843 (1983). A rich growth medium (YEPD) containing yeast extract peptone and dextrose (glucose) was used for non-selective yeast growth A minimal growth medium (SD) containing dextrose and appropriate supplements was used for selective growth and scoring of nutritional markers. These media are described by Sherman et al., Methods in Yeast Genetics (Cold Spring Harbor Laboratory, New York, 1974).In experiments involving growth on galactose, 2% galactose was substituted for glucose. To add the selective agent sulfometuron methyl (molecular weight 364) to solid media, it was dissolved in acetone at 2.5mg/mL and added to media to obtain a final concentration of 30 µg/mL immediately prior to pouring into culture dishes.

DNA Preparation and Manipulations

Plasmid DNA was prepared from E. coli according to (1) a rapid method substantially similar to that described by Quigley and Holmes Anal. Biochem. 114:193 (1981), or (2) a cesium chloride density gradient method substantially similar to that disclosed by Davis et al., Advanced Bacterial Genetics: A Manual for Genetic Engineering (Cold Spring Harbor Laboratory, New York, (1980)). Yeast DNA was prepared by a method similar to that described by Davis et al. Methods in Enzymology, 65: Part I (Academic Press, New York, 1980).

Selected host strains of yeast were transformed by a method similar to that of Hinnen et al., Proc. Nat. Acad. Sci. USA 75: 1929 (1978), except for the following modification. Recipient cells were incubated with glusulase (a β-gluouronidase/sulfatase preparation) for 2 hours at 30° in 1M sorbitol containing 1% beta-mercaptoethanol and 0.1 M sodium citrate, pH 5.8, to form spheroplasts. E. coli strains were transformed according to (1) a method similar to that of Mandel and Higa, J. Mol. Biol. 53: 159 (1970) or (2) a method similar to that of Hanahan, J. Mol. Biol. 166: 557 (1983), when high efficiency was desired.

All other methods of manipulating DNA are described by Maniatis et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, New York, 1982).

Southern Analysis and DNA Sequencing

DNA was transferred to nitrocellulose membranes from agarose gels and probed with specific labeled DNA fragments according to a method similar to that described by Southern, J. Mol. Biol. 98: 503 (1975), herein referred to as "Southern analysis". DNA segments were sequenced, using a dideoxynucleotide procedure similar to that of Sanger et al., J. Mol. Biol. 143: 161 (1980).

The method of the present invention is further described by the following examples, wherein all parts and percentages are by weight and degrees as Celsius.

Example 1

Site-Specific Deletion of LEU2 Gene in Yeast on Chromosome 7

Site-specific deletion of a LEU2 gene present in yeast Saccharomyces cerevisiae DNA was effected according to the following procedure. Yeast strains which were auxotrophic for leucine because of a leu2 gene mutation were transformed with the following plasmids--(1) pBS49 carrying a cre gene under regulatory control of a GAL1 promoter and (2) pBS42 or pBS43 carrying a functional LEU2 gene flanked by loxP sites in the same orientation. The transformed yeast strain contained a functional LEU2 gene and could grow in the absence of leucine. Activation of the GAL1 promoter with galactose effected expression of the cre gene and deletion of the LEU2 gene. The resulting yeast strain was dependent on leucine for growth.

Construction of pBS39 and pBS49

A flow chart illustrating the method used for construction of plasmids pBS39 and pBS49 is set forth in Figure 1. Plasmid pBS7, a portion of which is shown in Figure 1, is a derivative of plasmid pRH103 which contains the Δ6 deletion. Plasmid pBS7 differs from pRH103 in that the first DNA sequence - ATG - encountered on the cre coding strand of pBS7 starting at the Xho I site is that of an intact cre gene. Plasmid pBS7 also has a promoter which controls expression of the cre gene in E. coli. Plasmid pBS7 was digested with Xho I and a Bgl II-Xho I adapter (DNA sequence: TCGAGTAGATCTAC) was ligated to the digested plasmid. The resulting construct was then digested with Bgl II and Sal I. The digestion generated a cre containing fragment which was purified and then ligated to plasmid pBM150, described by Johnston and Davis, Mol. Cell. Biol. 4: 1440 (1984). The resulting plasmid, designated pBS39, was an autonomously replicating centromere containing yeast vector having a cre gene under control of a GAL1 promoter

Plasmid pBS49 containing the cre gene under the control of the GAL1 promoter was derived from plasmid

pBS39 according to the method shown in Figure 1. The Xho 1 - Sal I fragment of psS7 containing the cre gene was inserted into the Xho I site present in mouse metallothionein gene MT-1, described by Pavlakis and Hamer, Proc. Nat. Acad. Sci. USA 80:397 (1983). The resulting plasmid, designated pBS31, contained a cre gen upstream from the mouse MT-1 gene and, in particular, a 3' region of th MT-1 gene which contained a polyadenylation signal. The Eco RI site at the 3' end of the MT-1 gene was converted to a Sal I site and the resulting Sal I-Bam HI fragment was inserted into pBS39 which had been digested by Sal I and Bam HI, to form pBS49.

Plasmid pBS49 shares with pBS39 the ability to replicate autonomously in E. coli and yeast. Both plasmids have a cre gene under control of a GAL1 promoter In addition, pBS49 contains a mammalian polyadenylation signal, provided by the MT-1 gene, located 3' to the cre gene. The polyadenylation signal may facilitate expression of the cre gene in other eucaryotic cells. However, DNA sequences derived from MT-1 are unnecessary for cre gene expression from plasmid pBS49 in yeast, as shown below.

Construction of pBS42 and pBS43

A flow chart illustrating the method used for construction of plasmids pBS42 and pBS43 is set forth in Figure 2. A LEU2 gene flanked by loxP sites in the same orientation was obtained from plasmid pRH499 according to the following method. The Hind III site was removed from plasmid pRH499 to form plasmid pBS30. The 6.1 kilobase (kb) Hind III fragment of pJM53 is homologous to a region located between TRP5, a gene having a known location on chromosome 7 and required for tryptophan biosynthesis, and the LEUI gene also having a known location on chromosome 7. This fragment was self-ligated and digested with Xho I to produce a fragment joined head to tail. The fragment was then inserted into the Xho I site of pBS30 in both orientations t produce pBS42 and pBS43. The segment of DNA from chromosome 7 present on pJM53 was included t direct the resulting plasmid to a homologous region on a yeast chromosome by the yeast's endogenous recombination system.

Transforming Yeast with pBS42 and pBS43

Plasmids pBS42 and pBS43 were linearized with Hind III and transformed into yeast strain DBY931,which contains a leu2 mutation. Yeast cells that did not require leucine for growth were selected. Figure 3 shows that integration of these plasmids into chromosome 7 results in a leu2 gene flanked by loxP sites. The orientation of the lox sites relative to the centromere depends on whether pBS42 or pBS43 was the transforming plasmid. Integration of pBS42 generates yeast strain BSY4 having a substrate chromosome with loxP sites pointing away from the centromere of chromosome 7 as shown in Figure 3A. Integration of pBS43 generates yeast strain BSY16 having the loxP sites pointing toward the centromere of chromosome 7 as shown in Figure 3B.

Transforming Yeast with pBS49

The cre gene was then introduced into the yeast strains transformed with plasmids pBS42 and pBS43 according to the following procedure. Yeast strain DBY745, containing mutant genes ura3 and leu2, was transformed with plasmid pBS49 which carries a functional URA3 gene. Transformed yeast not requiring uracil for growth were selected, and designated yeast strain BSY3. Yeast strain BSY4 which contains the loxP substrate on its chromosome 7 was then mated with yeast strain BSY3 which contains plasmid pBS49 having cre gene under the control of the GAL1 promoter. This mating generated a diploid yeast strain designated BSY38. As a control, the isogenic diploid yeast strain BSY63 was constructed which differs from yeast strain BSY38 only in that it lacked plasmid pBS49. Similarly, yeast strain BSY16 was mated with yeast strain BSY3 to produce a diploid yeast strain designated BSY45 which contained both a cre gene and a modified chromosome 7. Yeast strain BSY16 was also mated with yeast strain DBY745 to produce the isogenic control strain BSY70 which lacked plasmid pBS49 and therefore the cre gene.

Effecting Production of the cre Gene Product

St. John and Davis, Cell 16: 443 (1979), disclose that the GAL1 promoter is inactive in cells growing on glucose but is induced to a 1000 fold greater activity in the presence of galactose. The strains shown in Table I were grown on plates containing either glucose or galactose. The resulting colonies were replicated t selective media to determine whether or not they required leucine for growth (a Leu- phenotype). The results are shown in Table I.

TABLE 1

Deletion of LEU2 Gene

Yeast Strain	Plasmid with lox sites	Plasmid with cre gene	Carbon Source	Colonies requiring leucine	Total Colonies
BSY38	pBS42	pBS49	glu	0	610
BSY38	pBS42	pBS49	gal	610	610
BSY63	pBS42	none	glu	0	86
BSY63	pBS42	none	gal	0	80
BSY45	pBS43	pBS49	glu	0	77
BSY45	pBS43	pBS49	gal	100	100
BSY70	pBS43	none	glu	0	80
BSY70	pBS43	none	gal	0	100

glu = glucose gal = galactose

Yeast strains transformed with plasmid pBS49 carrying the cre gene, became leucine requiring (Leu-) when grown on galactose but not when grown on glucose. Yeast strains lacking plasmid pBS49 showed a completely stable non-leucine requiring (Leu+) phenotype. The results demonstrate that the gene product of the cre gene (1) can be expressed under the control of the GAL1 promoter, (2) is able to enter the yeast nucleus after translation in the yeast cytoplasm, and (3) does effect the recombination between two lox sites inserted into yeast DNA. Moreover, the recombination at lox sites occurs with sites having both orientations with respect to the centromere. The orientation does not affect the accessibility of the lox sites by the gene product of the cre gene.

The recombination event was shown to be efficient. A log phase culture of yeast strain BSY38 was grown with glucose as the carbon source then transferred to growth medium containing galactose. Aliquots of the yeast were removed from the medium containing galactose at the time intervals shown in Figure 4 and plated on a non-selective medium containing leucine and glucose. The resulting colonies were assayed by replica plating to a selective medium without leucine. The resulting plates were scored after one day and the results are shown in Figure 4. The presence of yeast requiring leucine for growth was detected 8 hours after induction with galactose. After 24 hours 98% of the initial culture had deleted the LEU2 gene as shown by this assay.

Physical evidence was obtained to demonstrate that the gene product of the cre gene produced recombination at the lox sites located on chromosome 7. Eight independent leucine requiring isolates of yeast strain BSY38 were obtained by plating BSY38 on agar medium containing leucine and galactose. The total DNA from each of these leucine requiring isolates was digested with Eco RI and the structure of the region at which pBS42 had integrated into chromosome 7 was determined by the method of Southern, J. Mol. Biol. 98: 503 (1975) using plasmid pBS78 as a probe. Plasmid pBS78 is derived from pBS42 by cre mediated recombination at the lox sites in an E. coli stain. Plasmid pBS78 contains sequences homologous to the Amp^R gene of pBR322 and to the segment of chromosome 7 DNA derived from pJM53 but lacks homology with the LEU2 gene of yeast. Homology detected by pBS78 is indicated in Figure 5 by the solid black bar. Figure 5 shows the analysis of seven of these leucine requiring derivatives. Shown are the haploid parent DBY931 (lane 1), the haploid BSY4 with the LEU2 containing substrate chromosome 7 (lane 2), the diploid BSY63 which lacks the cre plasmid pBS49 (lane 3), the diploid BSY38 with plasmid pBS49 (lane 4), and seven independent galactose induced leucine requiring derivatives of BSY38 (lanes 5-11). Also shown are the marker plasmids pBS78 and pBS42. The analysis shows that all leucine requiring derivatives lost the 3.4 kb fragment of DNA detected by the probe. Instead, leucine requiring derivatives are shown in Figure 5 to have a 1.8 kb fragment of DNA as predicted by a deletion of the LEU2 gene. The derivatives all show exactly the same structure indicating deletion had occured only on the modified chromosome 7 and only at the lox sites. To further show that the

specific deletion had occurred, integrated plasmid DNA from each of three of the leucine requiring isolates was recovered by cleaving the genomic DNA with Hind III. The DNA from each isolate was re-ligated and used to transform E. coli. The region proximate to the lox site was sequenced for two of the plasmids. The sequences were found to be identical to that predicted by recombination at the lox sites. The third plasmid was found to be identical to the other two by restriction mapping, but no sequencing was conducted.

Example 2

Site-Specific Deletion of LEU2 gene in Yeast

The following experiment shows that the recombination event at lox sites in yeast after galactose activation is dependent on a funtional cre gene. Plasmid pBS77, a derivative of plasmid pBS49 which contains a non-functional cre gene, was constructed according to the following procedure. Plasmid pBS49 was digested with Bam HI - which cuts within the cre gene -and the resulting staggered ends were made flush using the Klenow fragment of DNA polymerase I. The resulting DNA was religated to form plasmid pBS77 which is identical to pBS49 except that it contains a mutant cre gene which is inactive in E. coli. Diploid yeast strains BSY91 and BSY93 were constructed according to the following procedure. Yeast strain DBY745 was transformed with pBS77 and yeast cells able to grow in the absence of uracil were selected. The resulting yeast strain BSY92 was mated with BSY4 to produce BSY93. Similarly, yeast strain DBY745 was also transformed with plasmid pBS39-identical to pBS49 except it lacks the mouse MT-1 DNA sequences - to yield a yeast strain designated BSY90. Yeast strain BSY90 was mated with BSY4 to generate a diploid yeast strain designated BSY91.

The yeast strains shown in Table II were grown on agar medium containing galactose and leucine. Individual colonies were transferred onto agar medium containing glucose and leucine and were then tested for their ability to grow in the absence of leucine by replica plating to appropriate plates. The results are shown in Table-II.

TABLE II

Deletion of LEU2 Gene

Yeast Strain	Plasmid with lox sites	Plasmid with cre gene	Colonies requiring leucine	Total Colonies
BSY38	pBS42	pBS49	10	10
BSY91	pBS42	pBS39	10	10
BSY93	pBS42	pBS77	0	10
BSY63	pBS42	none	0	10

Table II shows that the presence of galactose does not affect deletion of the LEU2 gene in yeast transformed with pBS77 containing the mutant cre gene. Table II also shows that pBS39, which lacks the portion of the mouse metallothionein gene present in pBS49, is capable of expressing the cre gene and affecting recombination at lox sites in yeast DNA. Therefore, no portion of the MT-1 gene is required for expression or function of the cre gene in yeast transformed with plasmid pBS39.

Example 3

Site-Specific Deletion of LEU2 Gene in Yeast on Chromosome 13

This example demonstrates that Cre mediated recombination at lox sites can occur on a yeast chromosome other than Chromosome 7. Plasmids for inserting a DNA sequence comprising the LEU2 gene flanked by lox sites at the ILV2 locus on chromosome 13 were constructed according to the following method. An allele of ILV2 which codes for sulfometuron methyl resistance is present on plasmid pCP2-4-10 which is disclosed and described by Falco and Dumas, Genetics 109: 21 (1985). Plasmid pCP2-4-10 is deposited in the American Type Culture Collection and bears deposit accession number 39606 The Cla I and Hind III sites flanking the ILV2 gene on pCP2-4-10 were converted to Xho I sites. The fragment resulting from digestion with Xho I was inserted into the Xho I site of pBS30 to form plasmids pBS44 and pBS47 which differ only in the orientation of the inserted Xho I fragment containing the ILV2 gene. These two plasmids were integrated into chromosome

13 by transforming yeast strain DBY931 and selecting for non-leucine requiring transformants according t a procedure similar to that described in Example 1. Integration of plasmid pBS44 into the ILV2 locus on chromosome 13 yielded yeast strain BSY23. Integration of plasmid pBS47 into the ILV2 locus resulted in yeast strain BSY27. Non-leucine requiring transformants obtained by transforming yeast cells with pBS44 - such as yeast strain BSY23-differ from those obtained by transforming them with pBS47 - such as yeast strain BSY27 - in that the inserted LEU2 gene has flanking lox sites in opposite orientations with respect to each other, as shown in Figure 6. The structures of these chromosomes were verified by Southern analysis. Diploid strains of yeast containing one of these chromosomes, and pBS49 which provided a cre gene under control of GAL1 promoter were constructed by 1) mating BSY23 with BSY3 to generate yeast strain BSY31 and also with yeast strain DBY745 to generate the Cre- control yeast strain BSY56, and 2) mating BSY27 with BSY3 to generate yeast strain BSY35 and also with DBY745 to generate the isogenic Cre- control yeast strain BSY59.

The yeast strains shown in Table III were grown on agar medium containing galactose and leucine. Individual colonies were transferred onto agar medium containing glucose and leucine and were then tested for their ability to grow in the absence of leucine by replica plating to appropriate plates. The results are shown in Table III.

TABLE III

Deletion of LEU2 Gene

Yeast Strain	Plasmid with lox sites	Plasmid with cre gene	Colonies requiring leucine	Total Colonies
BSY31	pBS44	pBS49	5	5
BSY56	pBS44	none	0	5
BSY35	pBS47	pBS49	5	5
BSY59	pBS47	none	0	5

Table III shows that transformed strains which contain a cre gene delete the LEU2 gene when grown on galactose containing medium. Thus, the gene product of the cre gene is able to recombine lox sites in either orientation with respect to normal sequence of chromosome 13 to generate chromosomal deletions on chromosome 13 as well as on chromosome 7.

Example 4

Site-Specific Inversion of LEU2 Gene in Yeast

Site-specific inversion of LEU2 gene present in yeast DNA is effected according to the following method. A first plasmid containing a cre gene under control of the GAL1 promoter is constructed according to a method similar to that used to construct pBS39 and pBS49 as shown in Figure 1. A second plasmid containing a selectable marker such as sulfometuron methyl resistance and a LEU2 gene flanked by loxP sites is constructed according to a method similar to that used to construct pBS44 and pBS47 except that the LEU2 gene is inserted into the plasmid (1) without a promoter, (2) with flanking loxP sites in opposite orientations with respect to each other and (3) with 3' end of the LEU2 gene proximate to a nucleotide regulatory sequence such that the gene is transcribed in an antisense direction.

A yeast strain which is auxotrophic for leucine is transformed with both plasmids according to a method similar to that described in Example 1 and Example 3. The resulting yeast are grown in a media containing glucose and leucine. The yeast require leucine to grow since the LEU2 gene is inverted with respect to its promoter The GAL1 promoter is activated by the presence of galactose as described in Example 1 thereby effecting expression of the cre gene and producing the inversion of the LEU2 gene. The resulting yeast are capable of growing in the absence of leucine.

Claims

I. A strain of yeast transformed with the following DNA sequences:

i) a first DNA sequence comprising a regulatory nucleotide sequence and a cre gene,
ii) a second DNA sequence comprising a first lox site, and
iii) a third DNA sequence comprising a second lox site.

2. A strain of yeast as claimed in claim 1, wherein the second and third DNA sequences are connected by a pre-selected DNA segment.

3. A strain of yeast as claimed in claim 2, wherein the pre-selected DNA segment is a gene for a structural protein, an enzyme, or a regulatory molecule.

4. A strain of yeast as claimed in any one of the preceding claims wherein the first and second lox sites are loxP sites.

5. A strain of yeast as claimed in any one of the preceding claims wherein the cre gene is isolated from bacteriophage P1.

6. A strain of yeast as claimed in any one of the preceding claims wherein the regulatory nucleotide sequence is a GAL1 promoter.

7. A plasmid having a regulatory nucleotide sequence and a cre gene, said plasmid being capable of transforming yeast.

8. The plasmid pBS39.

9. A plasmid having two lox sites connected by a pre-selected DNA segment, said plasmid being capable of transforming yeast.

10. The plasmid pBS44.

11. A microorganism transformed with a plasmid as claimed in any one of claims 7 to 10.

12. A strain of yeast transformed with a plasmid as claimed in claim 7 or claim 8.

13. The strain of yeast deposited as ATCC 20772 and mutants thereof.

14. A strain of yeast transformed with a plasmid as claimed in claim 9 or claim 10.

15. The strain of yeast deposited as ATCC 20773 and mutants thereof.

16. A process for producing a diploid strain of yeast as defined in any one of claims 1 to 6 which comprises mating a strain of yeast as claimed in claim 12 or claim 13 with a strain of yeast as claimed in claim 14 or claim 15.

17. A method for producing site-specific recombination of DNA in yeast which comprises activating the regulatory nucleotide sequence of a strain of yeast as claimed in any one of claims 1 to 6 thereby effecting expression of the cre gene.

18. A method as claimed in claim 17 wherein the site-specific recombination of the yeast DNA results in the expression of an engineered gene.

Claims for the following Contracting States: AT,ES

1. A process for producing site-specific recombination of DNA in yeast, comprising:
a) introducing into the DNA the following DNA sequences:
i) a first DNA sequence comprising a regulatory nucleotide sequence and a cre gene,
ii)a second DNA sequence comprising a first lox site, and
iii) a third DNA sequence comprising a second lox site, and
b) activating the regulatory nucleotide sequence thereby effecting expression of the cre gene.

2. A process as claimed in claim 1, wherein the second and third DNA sequences are introduced into the DNA in yeast connected by a pre-selected DNA segment.

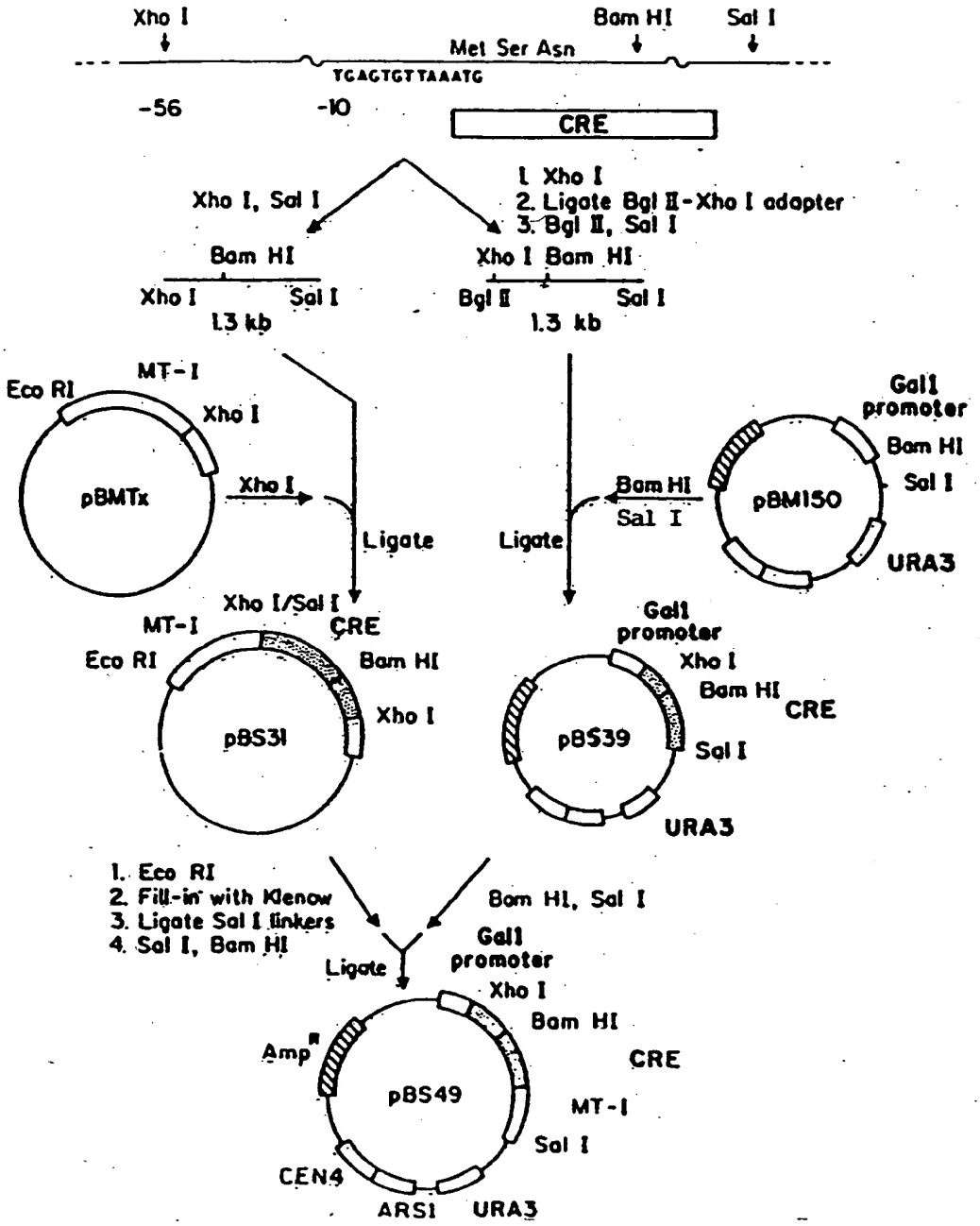
3. A process as claimed in claim 1 or claim 2, wherein the cre gene is isolated from bacteriophage P1.

4. A process as claimed in any one of the preceding claims, wherein the first and second lox sites are loxP sites.

5. A process as claimed in any one of the preceding claims, wherein the pre-selected DNA segment is a gene for a functional protein, an enzyme, or a regulatory molecule.

6. A process as claimed in any one of the preceding claims wherein the regulatory nucleotide sequence is a GAL1 promoter.

F I G. 1


Xho I
Bam HI
Sal I
Met Ser Asn
TGAGTGTTAAATG
-56
-10
CRE
Xho I, Sal I
1. Xho I
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3. Bgl II, Sal I
Bam HI
Xho I
Sal I
1.3 kb
Xho I Bam HI
Bgl II
Sal I
1.3 kb
Eco RI
MT-I
Xho I
pBMTx
Xho I
Ligate
Gal1 promoter
Bam HI
Sal I
pBM150
URA3
Bam HI
Sal I
Ligate
Xho I/Sal I
CRE
MT-I
Eco RI
Bam HI
Xho I
pBS31
Gal1 promoter
Xho I
Bam HI
CRE
pBS39
Sal I
URA3
1. Eco RI
2. Fill-in with Klenow
3. Ligate Sal I linkers
4. Sal I, Bam HI
Bam HI, Sal I
Ligate
Gal1 promoter
Xho I
Bam HI
CRE
Amp^R
pBS49
MT-I
Sal I
CEN4
ARS1
URA3

FIG. 2

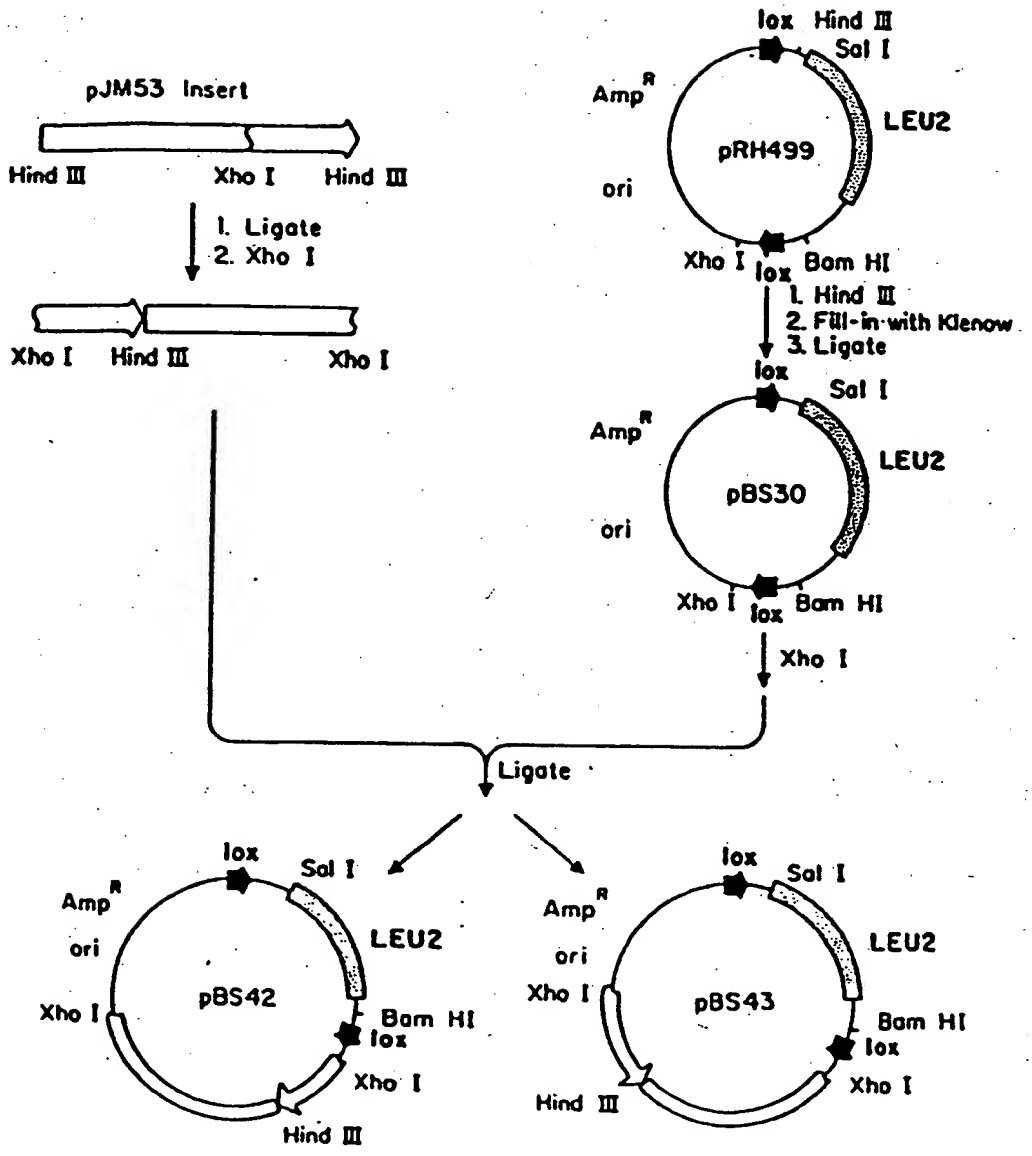

pJM53 Insert
Hind III
Xho I
Hind III
1. Ligate
2. Xho I
Xho I
Hind III
Xho I
lox
Hind III
Sal I
Amp^R
LEU2
pRH499
ori
Xho I
lox
Bam HI
1. Hind III
2. Fill-in with Klenow
3. Ligate
lox
Sal I
Amp^R
LEU2
pBS30
ori
Xho I
lox
Bam HI
Xho I
Ligate
lox
Sal I
Amp^R
ori
LEU2
Xho I
pBS42
Bam HI
lox
Xho I
Hind III
lox
Sal I
Amp^R
ori
LEU2
Xho I
pBS43
Bam HI
lox
Hind III
Xho I

FIG. 3

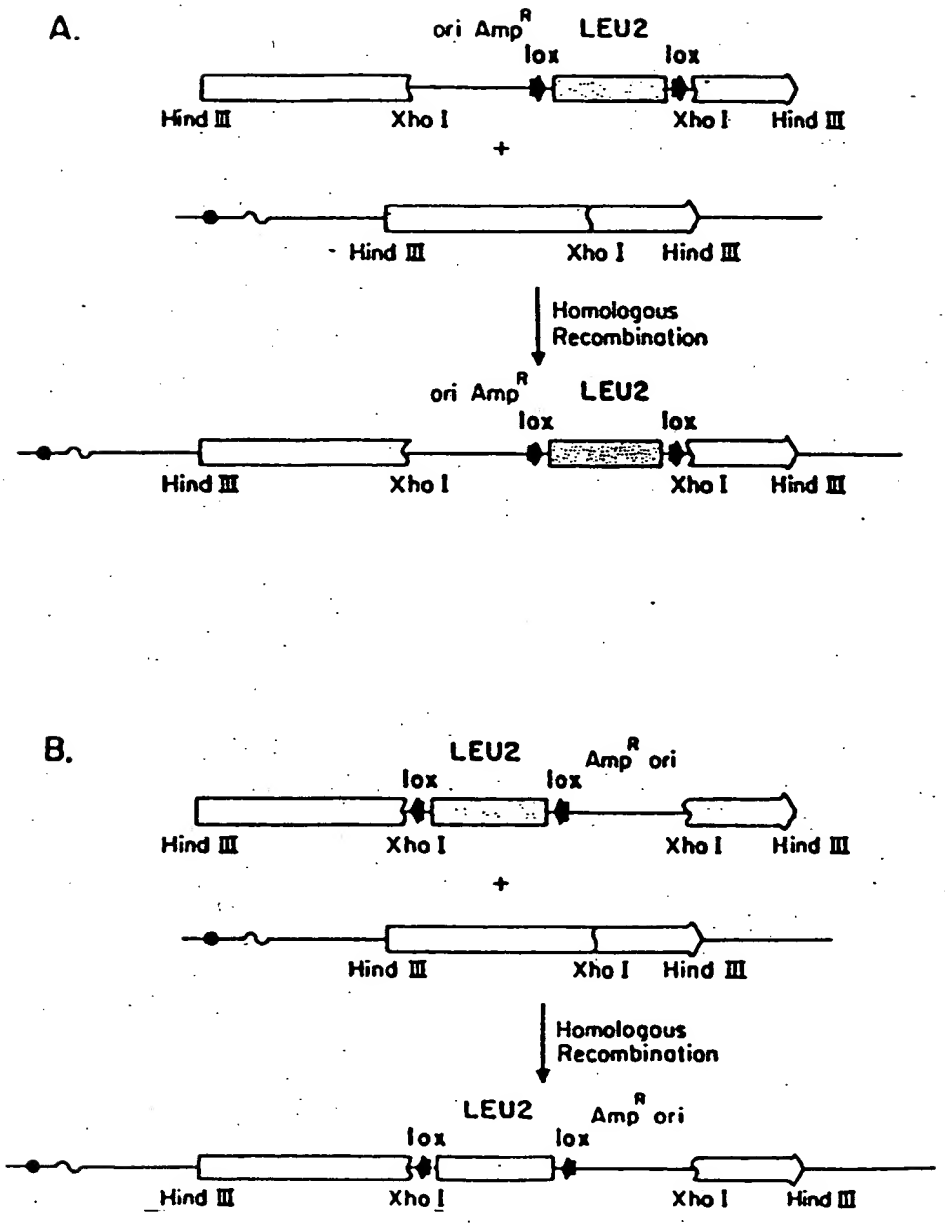

A.
ori Amp^R
LEU2
lox
lox
Hind III
Xho I
Xho I
Hind III
+
Hind III
Xho I
Hind III
Homologous Recombination
ori Amp^R
LEU2
lox
lox
Hind III
Xho I
Xho I
Hind III
B.
LEU2
Amp^R ori
lox
lox
Hind III
Xho I
Xho I
Hind III
+
Hind III
Xho I
Hind III
Homologous Recombination
LEU2
Amp^R ori
lox
lox
Hind III
Xho I
Xho I
Hind III

FIG. 4

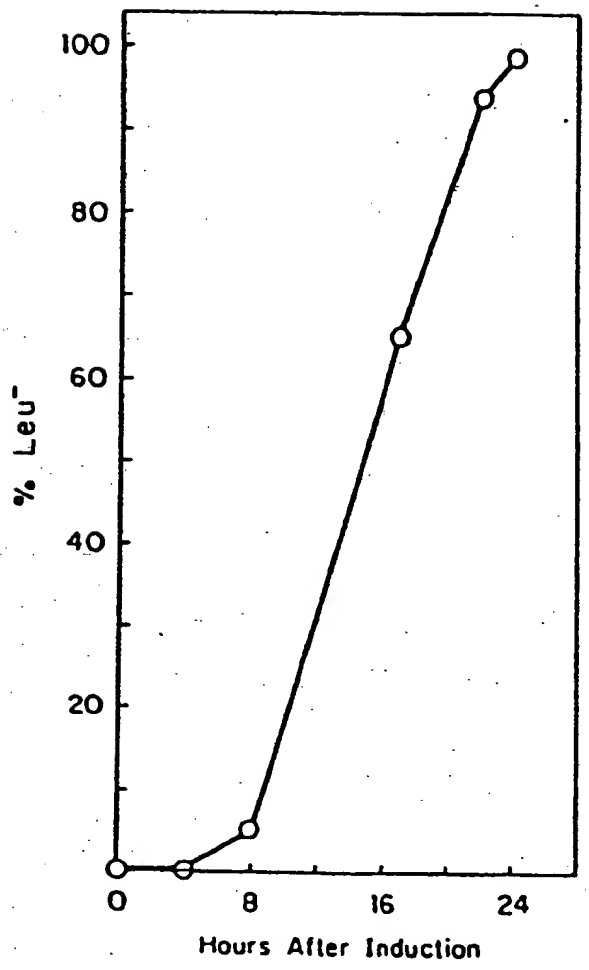


FIG. 5

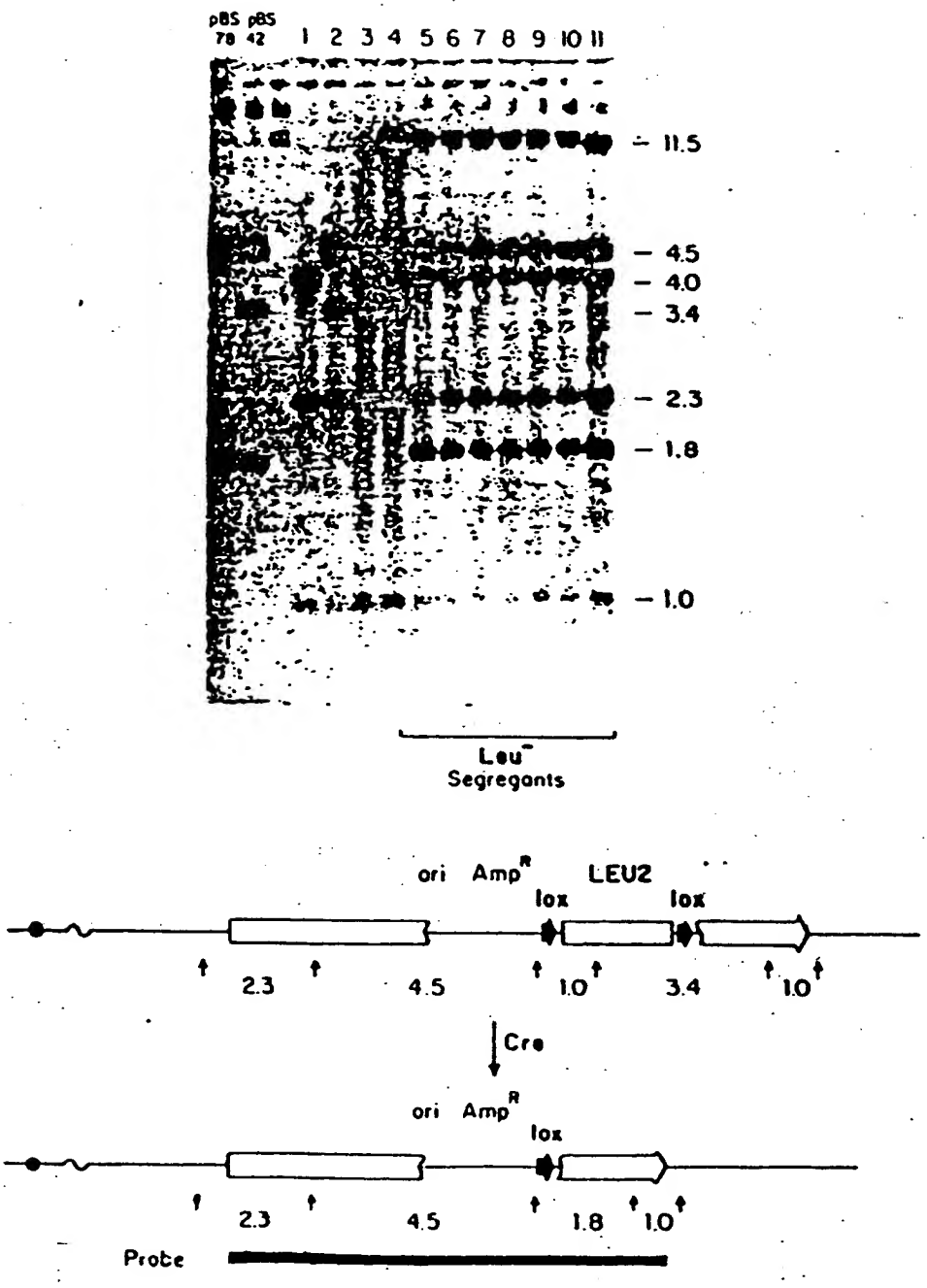
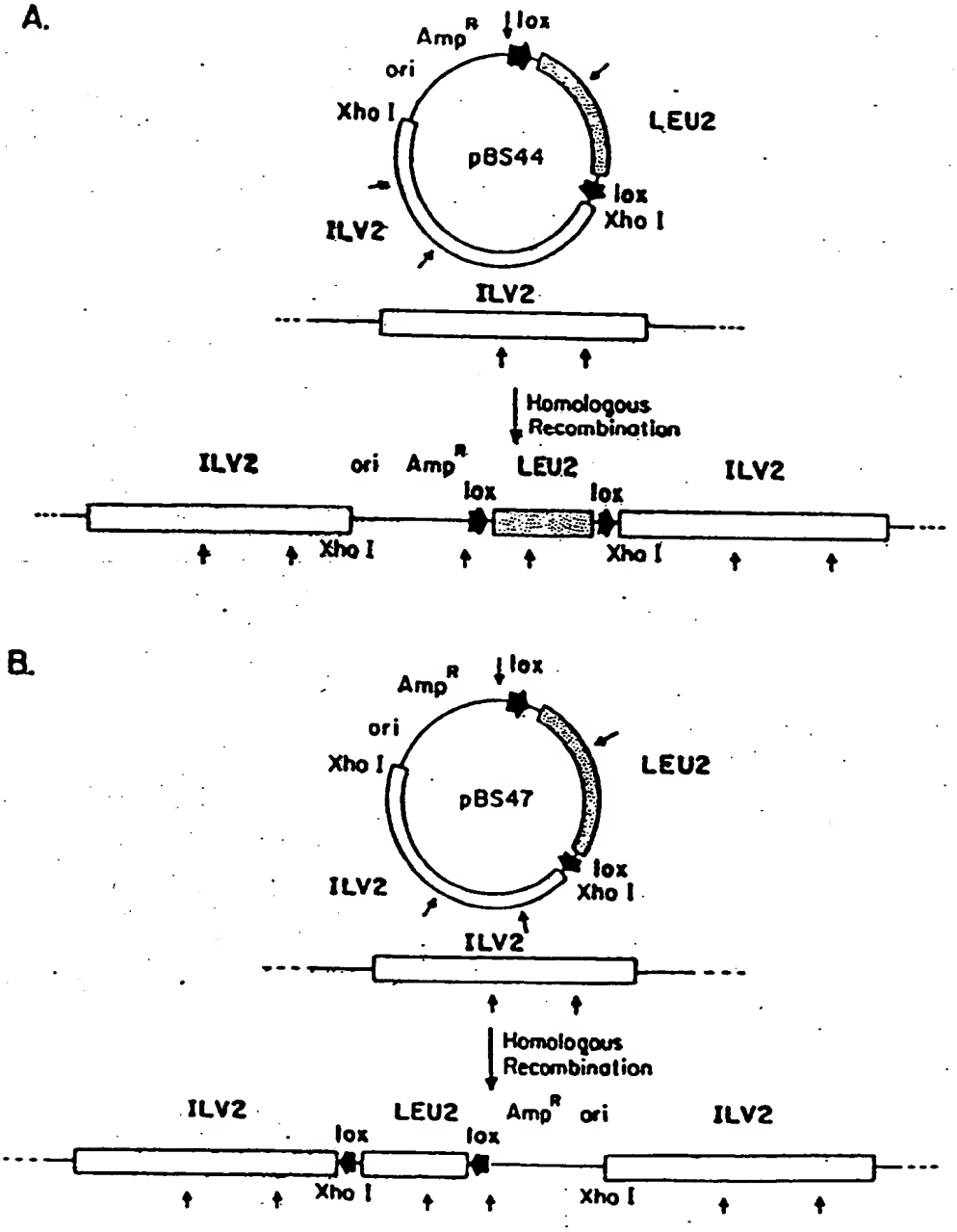

pBS 78
pBS 42
1 2 3 4 5 6 7 8 9 10 11
11.5
4.5
4.0
3.4
2.3
1.8
1.0
Leu⁻ Segregants
ori
Amp^R
LEU2
lox
lox
2.3
4.5
1.0
3.4
1.0
Cre
ori
Amp^R
lox
2.3
4.5
1.8
1.0
Probe

FIG. 6


A.
Amp^R
lox
ori
Xho I
LEU2
pBS44
lox
Xho I
ILV2
ILV2
Homologous Recombination
ILV2
ori
Amp^R
LEU2
ILV2
lox
lox
Xho I
Xho I
B.
Amp^R
lox
ori
Xho I
LEU2
pBS47
lox
Xho I
ILV2
ILV2
Homologous Recombination
ILV2
LEU2
Amp^R
ori
ILV2
lox
lox
Xho I
Xho I

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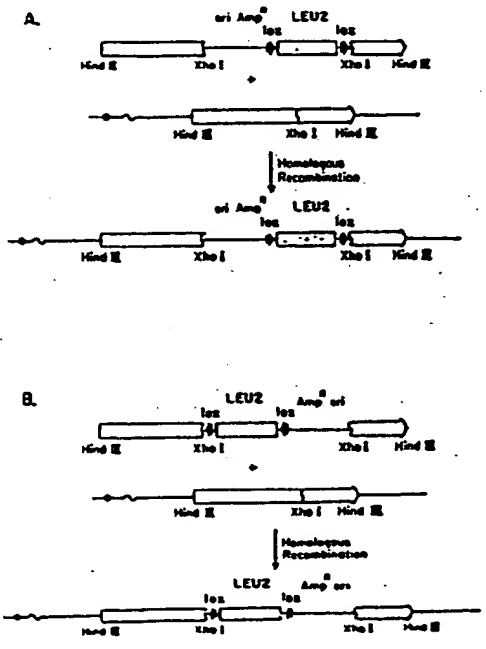
(54) Site-specific recombination of DNA in yeast.

(57) A method for producing site-specific recombination of DNA in yeast at regions designated lox sites is disclosed. Novel strains of yeast transformed with DNA sequences comprising two lox sites, a regulatory nucleotide sequence and a cre gene and yeast-transforming plasmids having a regulatory nucleotide sequence and a cre gene are also disclosed.

FIG. 3



EP 0 220 009 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 86 30 7703

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
P,X	JOURNAL OF CELLULAR BIOCHEMISTRY, supplement 10B, no. 10, Part B, 1986, page 242, A.R. LISS, New York, US; B. SAUER: "Expression and functioning in yeast of a bacterial site specific recombination system" ---	1-17	C 12 N 15/00 C 12 N 1/18
T	MOLECULAR AND CELLULAR BIOLOGY, vol. 7, no. 6, June 1987, pages 2087-2096, American Society for Microbiology; B. SAUER: "Functional expression of the cre-lox site-specific recombination system in the yeast saccharomyces cerevisiae" * Whole article * ---		
D,A	CELL, vol. 32, April 1983, pages 1301-1311, MIT, Cambridge, MA, US; K. ABREMSKI et al.: "Studies on the properties of P1 site-specific recombination: evidence for topologically unlinked products following recombination" * Whole article * -----	1	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	01-07-1988	HUBER-MACK A.

CATEGORY OF CITED DOCUMENTS

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